Sources of variability and comparability between salmonid stomach contents and isotopic analyses: study design lessons and recommendations

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Abstract: We compared sources of variability and cost in paired stomach content and stable isotope samples from three salmonid species collected in September 2001–2005 and describe the relative information provided by each method in terms of measuring diet overlap and food web study design. Based on diet analyses, diet overlap among brown trout, rainbow trout, and mountain whitefish was high, and we observed little variation in diets among years. In contrast, for sample sizes $n \geq 25$, 95% confidence interval (CI) around mean $\delta^{15}N$ and $\delta^{13}C$ for the three target species did not overlap, and species, year, and fish size effects were significantly different, implying that these species likely consumed similar prey but in different proportions. Stable isotope processing costs were US$12 per sample, while stomach content analysis costs averaged US$25.49 ± $2.91 (95% CI) and ranged from US$1.50 for an empty stomach to US$291.50 for a sample with 2330 items. Precision in both $\delta^{15}N$ and $\delta^{13}C$ and mean diet overlap values based on stomach contents increased considerably up to a sample size of $n = 10$ and plateaued around $n = 25$, with little further increase in precision.

Résumé : Nous avons comparé les sources de variabilité et les coûts dans des échantillons appariés de contenus stomacaux et d'isotopes stables récoltés en septembre 2001–2005 chez trois espèces de salmonidés; nous décrivons l'information relative apportée par chaque méthode en ce qui a trait au chevauchement des régimes alimentaires et au plan d'étude des réseaux trophiques. D'après les analyses des régimes alimentaires, le chevauchement est élevé entre la truite brune, la truite arc-en-ciel et le ménomini de montagnes et il y a peu de variation de régime entre les années. En revanche, pour des tailles d'échantillon de $n \geq 25$, les intervalles de confiance (IC) à 95 % autour des valeurs moyennes de $\delta^{15}N$ et de $\delta^{13}C$ pour les trois espèces ciblées ne se chevauchent pas et les effets de l'espèce, de l'année et de la taille du poisson sont significativement différents, ce qui implique que ces espèces consument vraisemblablement des proies semblables, mais dans des proportions différentes. Le coût de traitement des analyses d'isotopes stables est de 12 $US par échantillon, alors qu'une analyse de contenu stomacal coûte en moyenne 25,49 ± 2,91 $US (IC à 95 %) et varie de 1,50 $US pour un tube digestif vide à 291,50 $US pour un échantillon contenant 2330 proies. La précision du chevauchement moyen des régimes alimentaires basé sur les valeurs de $\delta^{15}N$ et de $\delta^{13}C$ et sur les contenus stomacaux augmente considérablement jusqu'à une taille d'échantillon de $n = 10$ et atteint un plateau vers $n = 25$ avec peu d'augmentation de précision par la suite.

[Traduit par la Rédaction]

Introduction

Collection and analysis of stomach content and stable isotope data both provide a means to describe trophic interactions among species or feeding guilds. This information has been used to quantify resource overlap between species (e.g., Schoener 1970; Vander Zanden et al. 1997), assess effects of fish management decisions (e.g., Harvey and Kitchell 2000), and evaluate the potential impacts of invasive species on natural food webs (e.g., Vinson and Baker 2008). Our ability to detect biologically relevant, statistical differences among trophic levels or between species diets is determined by the variability in the measurement around mean values and the number of replicate samples, as well as the complexity of the food web. Primary sources of variation in both stomach contents and stable isotopes are ontogenetic changes in diet (Genner et al. 2003), seasonal diet shifts (Vizzini and Mazzola 2003; Perga and Gerdeaux 2005), and variance in prey selection among individuals (Beaudoin et al. 1999).

Historically, the characterization of diets and trophic linkages among fish species relied primarily on evaluation of stomach contents (Hyslop 1980; Bowen 1983). The collection and analysis of stomach content data, however, has a

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number of drawbacks; it can be detrimental to the study animal; the material can be laborious to identify; and stomach contents can be highly variable based on variation in digestion rates, feeding habits, seasonal or diel collection times, fish size, and individual dietary whims (Bowen 1983). Traditional stomach content measurements include counts, frequency of occurrence, and volume or weight of individual prey items (Hyslop 1980). While a number of indices incorporating one of more of these measurements have been developed to describe the diets of individual species and to assess trophic position and diet overlap among species (Cailliet and Barry 1979; Hyslop 1980; Cortés 1997), there has been a lack of consistency in the application of statistical tests to analyze these metrics, which has hindered comparative studies (reviewed by Cortés 1997). The reporting of measures of variance associated with stomach content data appears particularly problematic. In a review of more than 200 diet studies, Ferry and Cailliet (1996) reported that none of the studies they reviewed provided any estimates of precision in describing diet composition.

In the last 30 years, stomach content analyses have been complemented or replaced by analysis of stable isotope ratios, principally nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$), for the determination of the relative trophic position of organisms or species (or "who eats whom"; Peterson and Fry 1987). In comparison with stomach content data, stable isotope data can provide longer-term dietary inferences, reduced labor in sample collection and preparation, and collection methods are typically nonlethal. Stable isotopes integrate dietary patterns over weeks, months, or even years (Hesslein et al. 1993) instead of hours or days for stomach contents. The integration of the dietary regime over time may reduce variation among individuals as compared with stomach contents and potentially provide more direct evidence for which diet items are most important in terms of actually being assimilated into tissue over time (Vinson and Baker 2008). Given the potential to improve our ability to infer trophic differences among species, this trait (time integration and assimilation) makes stable isotopes especially desirable in ecological studies (Vander Zanden et al. 1997; Harvey and Kitchell 2000; Kelly et al. 2004). In contrast with stomach content measures, which are often categorical or proportion data, stable isotope data are continuous numeric data facilitating the statistical detection of biologically relevant patterns (Hobson 1993).

For stomach contents, the occurrence of empty stomachs and our ability to identify partially digested prey can also increase variation in diet measurements. In a review of stomach content data from 254 fish species, Arrington et al. (2002) reported the number of empty stomachs averaged 16% and varied from 0% to 79% among individual species. The occurrence of unidentifiable prey in stomachs appears similar, but is strongly influenced by prey type, with planktivores and invertivores having more unidentifiable material in their stomachs than piscivores.

In addition to these primary sources of variation in both stomach contents and stable isotopes, additional error may occur for stable isotopes when associated with sample collection, preparation, or analytical measurement, and differences among tissue types within individual specimens have been documented. Variation in stable isotope values due to sample collection and preparation errors is likely minor (Jacob et al. 2005), as are analytical errors associated with isotope ratio mass spectrometry (Jardine and Cunjak 2005). In general, variation among fish tissues appears slight: McCarthy and Waldron (2000) — brown trout (*Salmo trutta*); Shannon et al. (2001) — humpback chub (*Gila cypha*); Finlay et al. (2002) — rainbow trout (*Oncorhynchus mykiss*); Jardine et al. (2005) — Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*); Sanderson et al. (2009) — Chinook salmon (*Oncorhynchus tshawytscha*). However, Suzuki et al. (2005) and Perga and Gerdeaux (2005) both measured differences among tissue types, and the difference or rate of isotopic change among tissues in response to changes in diet appears to be directly related to tissue growth (Hesslein et al. 1993). Thus, changes in isotopic composition in slow-growing fish or slow-growing tissue could take months or years. Overall, variation in stomach contents and stable isotopes appear most strongly influenced by ontogenetic changes, seasonal differences, and individual variation among individuals. Variation in diets due to ontogenetic changes may be reduced by stratifying analyses by fish size. Sampling within a discrete sampling period or stratifying analyses by season can reduce seasonal differences.

This study analyzed paired stomach content and isotopic samples from the same individuals to evaluate the effects of sample size on the ability to resolve dietary overlap and differences in isotopic signatures and subsequently to compare inferences about trophic structure made from each data type alone (stomach, isotopic data) vs. both data types together. We examined stomach contents and muscle isotopic signatures in three species of salmonids that function as top predators (brown trout, rainbow trout, mountain whitefish (*Prosopium williamsoni*)) in a stream ecosystem. Specifically, we investigated (*i*) variation in the stomach contents and $\delta^{13}C/\delta^{15}N$ values among individuals, among species, within years, and among years; (*ii*) the influence of sample size on variation and precision of mean dietary overlap and isotopic composition; and (*iii*) precision gained vs. monetary cost of processing additional diet or isotope data.

The overall goal of this study was to answer two very broad and important questions in applied ecology. (*i*) As compared with stomach content samples, what information do stable isotope samples provide and how many do you need? Within this context, (*ii*) what information do stomach content samples provide relative to stable isotope samples? Specifically, we capitalized on the opportunity to use a large data set of paired isotope samples and stomach content data to evaluate sources of variability and to aide in designing food web studies. For three salmonid species, we report (*i*) the variation in stomach contents and $\delta^{13}C$ and $\delta^{15}N$ values among individuals within and among years; (*ii*) variation in $\delta^{13}C$ and $\delta^{15}N$ values within individual fish and between samples collected from muscle tissue versus adipose fins; (*iii*) the influence of sample size on the magnitude of variation around mean diet overlap and $\delta^{13}C$ and $\delta^{15}N$ values among the three species attributed to interindividual differences; and (*iv*) we consider the implications of this variation for inferring trophic position from stable isotopes, a common goal of food web studies. We finish with a comparison of costs associated with processing stomach samples relative

to stable isotope samples, and a cost–benefit analysis of the precision gained with increasing sample size relative to the increasing cost of taking and analyzing more isotope samples.

Materials and methods

Study area

Fish and invertebrate samples were collected from the Green River downstream from Flaming Gorge Dam in northeastern Utah (40°54′N, 109°25′W). Vinson (2001) described the hydrology and biology of this reach in detail. The river can be characterized as clear and cold (2–14 °C annual temperature range), with deep runs (2–4 m) and pools (5–12 m), large cobble and boulder substrates, and abundant growth of aquatic plants that includes moss (*Amblystegium riparium*), *Cladophora glomerata*, *Chara* sp., *Elodea canadensis*, *Potamogeton crispus*, *Potamogeton pectinatus*, *Ranunculus* sp., and *Spirogyra* sp. Aquatic invertebrate assemblages are characteristic of a hypolimnetic release dam-influenced river and are dominated by Chironomidae, Simuliidae, Amphipoda, and *Baetis* mayflies (Vinson 2001). Fish populations are dominated by brown trout and rainbow trout, with fewer numbers of mountain whitefish and mottled sculpin (*Cottus bairdii*) present. Harju (2007) evaluated the potential influence of New Zealand mud snails (*Potamopyrgus antipodarum)* on the food web of this river reach using results from an isotope mixing model (Phillips and Gregg 2003) and a bioenergetics model and provides additional stable isotope data on other members of the food web not described here.

Field collections

Salmonid species and mottled sculpin were collected in proportion to their natural abundances by Utah Division of Wildlife personnel using electrofishing techniques for 2001–2005. Samples were collected each September from two ~10 km long stream reaches — just downstream from Flaming Gorge Dam and downstream from Little Hole — on two consecutive nights. All fish were collected at night between 20:00 and 23:00. Stomach contents were collected from 50 fish randomly selected at each location each year. Once collected, fish were anesthetized with tricaine methanesulfonate (MS-222), identified, measured to the nearest millimetre, weighed to the nearest 0.1 kg, and had their stomach contents removed by pulsed gastric irrigation (Light et al. 1983). Stomach contents were preserved in the field in 95% ethanol and returned to the laboratory. For stable isotope analyses, a single white muscle tissue sample was collected from below the dorsal fin with a 5 mm diameter dermal biopsy punch (Miltex Instrument Company, Bethpage, New York). The muscle plug was placed on ice for several hours and then frozen. The fish were then placed in a pen in the river to recover and then released. Aquatic invertebrates were collected for $\delta^{13}C$ and $\delta^{15}N$ analysis each September between 2001 and 2005 from the Green River in the same locations as the fish were collected. Invertebrates were collected qualitatively with kick nets, kept on ice in the field, and then frozen until processed.

Laboratory methods

Stomach contents

Stomach content samples were identified in the laboratory under a dissection microscope, generally to genus or species, and counted. Partially digested contents were identified and enumerated as conservatively as possible. For example, the presence of two invertebrate eyes was counted as one individual and unidentifiable fish bones were recorded as a single undetermined fish. Stomach content data were summarized into 10 major categories: Amphipoda (mostly *Hyalella azteca* and some *Gammarus lacustris*); Coleoptera (mostly Elmidae, *Optioservus*); Diptera (mostly Chironomidae and Simuliidae); Ephemeroptera (mostly *Baetis tricaudatus*); Mollusca (mostly Gastropoda, *Potamopyrgus antipodarum*); and others (Plecoptera (*Hesperoperla pacifica*), Trichoptera (mostly Hydroptilidae, *Hesperophylax*, and *Rhyacophila*), and Annelida (mostly Oligochaeta)), terrestrial invertebrates, deer mice (*Peromyscus maniculatus*), mottled sculpin, juvenile salmonids, and salmonid eggs.

Stable isotopes

Stable isotope samples were processed within a few weeks of collection. Invertebrates were identified to genus or species prior to processing. Individual invertebrates were cleaned of debris by rinsing them with deionized water. Snails were removed from their shells. All stable isotope material was dried for at least 48 h at 65 °C (Midwood and Boutton 1998), ground to a fine powder with a mortar and pestle, and packed in 8 mm × 5 mm tin capsules. Two milligrams of plants, invertebrates, and fish were used for isotopic analysis. When possible, one invertebrate was used to meet the mass requirement; if this was not possible, multiple organisms of the same taxa were pooled to meet the minimum requirement. Individual fish tissue samples were analyzed separately. $\delta^{13}C$ and $\delta^{15}N$ content was determined using a Europa Hydra 20/20 continuous flow isotope ratio mass spectrometer at the University of California–Davis Stable Isotope Facility (Davis, California, USA). Stable isotope contents are reported in del (δ) units, in parts per thousand (‰) relative to the reference standards for PeeDee belemnite for ^{13}C and nitrogen gas in ambient air for ^{15}N, calculated as follows:

$$\delta^{13}C \text{ or } \delta^{15}N = [(\text{Rsample}/\text{Rstandard}) - 1] \times 1000$$

where $R = {}^{13}CO_2/CO_2$ for $\delta^{13}C$ or $R = {}^{15}N/N_2$ for $\delta^{15}N$.

Data analysis

To reduce variation associated with ontogenetic changes in diet and best address our study design-based objectives, we included only salmonids > 250 mm or a size threshold that corresponds to fish age-2 or older. Previous work on this system demonstrated that trout smaller than 250 mm are invertivores, and larger fish are omnivores and piscivores (M.R. Vinson, unpublished data). Our analyses were based on samples collected from 139 brown trout, 33 mountain whitefish, and 111 rainbow trout. The number of salmonids from which paired stomach and isotope samples were collected from each year was 22 in 2001, 72 in 2002, 66 in 2003, 61 in 2004, and 62 in 2005. Brown trout mean total length was 415 mm and mean weight was 0.8 kg (range

250–673 mm, 0.2–3.0 kg). Mountain whitefish mean total length was 451 mm and mean weight was 1.2 kg (range 328–516 mm, 0.5–1.7 kg). Rainbow trout mean total length was 362 mm and mean weight was 0.5 kg (range 250–465 mm, 0.2–1.3 kg). For fish > 250 mm, length and weight did not vary substantially among years for any species. In 2005, three replicate muscle plugs and an adipose fin tissue sample were collected from each of five individual fish of each species. Stable isotope data were also collected from 26 mottled sculpin (27–105 mm), included only for trophic comparisons in the food web analysis described below.

Stomach contents

Nonmetric multidimensional scaling (MDS) was used to examine differences in stomach contents among salmonid species (Primer-E, ver. 6.1.12, Primer-E Ltd., Plymouth, UK). Count data were standardized to the maximum values, so samples were expressed as the percent composition of each prey category following Clarke and Gorley (2006). We used ordinations based on Sorenson/Bray–Curtis similarity distance measurements to provide graphical representations of assemblage patterns. In two-dimensional ordination, samples that group close to one another indicate similar assemblages, whereas samples far apart indicate relatively dissimilar assemblages. Significance of a priori groupings, species and years, were tested with a nested analysis of similarities (ANOSIM) using Primer ver. 6, where species was the main factor being tested, and year was nested within species. We calculated similarity percentages (SIMPER) in Primer to determine if a particular diet category varied widely among salmonids. In this test, the statistic R is a measure of effect size, where $R = 1$ indicates that samples within a group are more similar to each other than members from other groups, and $R = 0$ indicates that within-group similarity is equal to among-group similarity; an R near 1 indicates strong grouping, whereas an R near 0 indicates weak grouping.

We used Schoener's overlap index (Schoener 1970) to evaluate the effect of sample size on precision and mean diet overlap among species. Schoener's index was calculated as

$$\propto = 1 - 0.5\sum_{i}^{n} p_{x,i} - p_{y,i}$$

where $\propto$ is the overlap index, $p_{x,i}$ is the percentage of food type i eaten by species x, $p_{y,i}$ is the percentage of food type i eaten by species y, and n is the total number of prey item categories. Values range from 0 (no overlap) to 1 (complete overlap), and overlap is generally considered to be biologically significant when the value exceeds 0.60 (Zaret and Rand 1971). Effect of sample size on diet overlap values was evaluated by randomly selecting fish in sample size bins ranging from $n = 2$ to $n = 30$ and calculating index values based on these fish, repeated 1000 times for each sample size bin with replacement. We then calculated the mean and 95% confidence interval (CI) for each set of random draws.

Stable isotopes

To verify there were no significant intraindividual variation in isotopic signatures, we compared variation in isotopic composition among repeated samples taken within an individual fish (muscle, $n = 3$ for each sample) and the difference and variation between isotopic composition of muscle tissue samples versus tissue samples collected from the adipose fin ($n = 5$ for each tissue type) for each fish species. We then compared mean $\delta^{13}C$ and $\delta^{15}N$ between adipose and mean muscle tissue using pairwise two-sample t tests assuming unequal variance, with statistical significance based on an a priori $\alpha = 0.05$. Among replicated muscle tissue samples ($n = 3$ per fish), we evaluated the average difference in mean $\delta^{13}C$ and $\delta^{15}N$ values among fish and used nonoverlapping 95% CI as an indication of likely significant biological difference (Cumming 2009).

Isotopic variation among individuals and precision versus sample size

We evaluated variation in mean isotopic composition with a general linear model fit to both $\delta^{13}C$ and $\delta^{15}N$ as response variables and both categorical (species and year) and continuous (fish length) predictor variables, including interactions among variables (SAS Institute Inc. 2005). Because of heterogeneous variances among the species, the best model estimated a separate residual variance for each species. Fish length was mean-centered for each species prior to analysis, and we screened all possible interactions involving fish length to determine which were supported by data patterns for both $\delta^{13}C$ and $\delta^{15}N$, using Akaike's information criterion (AIC) to select the most parsimonious model (Burnham and Anderson 1998). Data analyses were obtained using the MIXED procedure in SAS/STAT software (version 9.2) in the SAS System for Windows. AIC values for comparing models that differed in fixed-effects specifications were obtained using the maximum likelihood method.

To evaluate the effect of sample size on the precision with which mean isotopic composition was estimated, we fit an analysis of variance of a two-way factorial in a completely randomized design to both $\delta^{13}C$ and $\delta^{15}N$ as response variables with species, year, and their interaction as categorical predictor variables, specifying heterogeneous variances for species (i.e., a separate residual variance was estimated for each species). The residuals from these models were then used in a bootstrapping exercise to assess the relationship between sample size and precision. Using this approach, the potentially confounding effect of year is factored out, and the analysis focused on the effects of sample size given the effects of variation in fish size on statistical and biological inference. Our evaluations were based on 1000 bootstrap samples of the data for each sample size (increments of 1 for $n = 2$–10, increments of 5 for $n = 10$–50). We set α for confidence limits at 0.05 (95% CI) and applied the bootstrap exercise to each species for both $\delta^{13}C$ and $\delta^{15}N$. Data analyses were obtained using the MIXED procedure in SAS/STAT software (version 9.2), and the %JACKBOOT macro, available from the SAS Web site (http://support.sas.com/kb/24/982.html, accessed 26 June 2010).

Food web and trophic overlap

The effect of sample size differences on the overall depiction of the food web and the degree of diet overlap among species or trophic groups was inferred by the degree of over-

lap in 95% CIs around mean values for different sample sizes. Diets and trophic positions were considered different when 95% CIs did not overlap (Cumming 2009). For lower trophic levels (sculpin, $n = 26$; salmonid eggs, $n = 13$; invertebrate predators, $n = 65$; detritivores, $n = 102$; and herbivores $n = 65$), the mean was determined from a randomly selected set of 25 samples from each lower trophic level (13 for salmonid eggs). This mean was then held constant for a sample size of 25 (as the purpose here is to show the effect of increasing precision), and 95% CIs were calculated based on the mean standard deviation of 100 random drawn samples with replacement for sample sizes of $n = 3$, 5, 10, and 25. Mean and 95% CI for brown trout, rainbow trout, and mountain whitefish were based on the bootstrap analysis described above. Invertebrate species were grouped into predators (principally Diptera (Tabanidae, Muscidae), Odonota (Coenagrionidae), Trichoptera (Rhyacophilidae, *Rhyacophila*)), detritivores (Amphipoda (*Gammarus lacustris* and *Hyalella azteca*), Diptera (Orthocladiinae, *Simulium*), Ephemeroptera (*Baetis tricaudatus*)), and herbivores (Coleoptera (*Optioservus*), Ephemeroptera (*Ephemerella*), Gastropoda (*Potamopyrgus antipodarum* and *Physella*)) based on dietary information in Thorp and Covich (1991) and Merritt et al. (2008).

Costs

Finally, we compared the costs of processing stomach content and stable isotope samples and evaluated the relative benefit of preparing and analyzing more isotope samples in terms of cost versus the increase in precision gained by adding additional samples. Costs for processing stomach content samples were based on the time required to process each sample. A labor cost of $10 per hour (all currency in US$) was used for sorting these samples into gross taxonomic categories (essentially invertebrate orders), $20 per hour for enumerating and identifying the individuals to genus or species, and $15 per hour for entering these data into a computer program. We estimated the cost for stable isotope samples at $12 per sample based on an analysis cost of $8 per sample for $\delta^{13}C$ and $\delta^{15}N$, the price we paid for these analyses over the 5-year study, a laboratory preparation cost of $3 per sample, and an estimated material supply cost of $1 per sample. Data are provided by the stable isotope analysis lab in electronic format, so no data entry cost was included for isotopes. On average, about 0.25 h of technician time is required per sample to dry, crush, and place the material into tin capsules. Supplies include disposable items such as biopsy punches and tin capsules as well as capital items such as a mortar and pestle, an electronic balance, and a drying oven. We did not include the cost for collecting fish in the field, as these costs will vary dramatically across different species and systems, and they are similar whether you are collecting stomach samples or stable isotope samples from these fish. We compare the mean cost for processing stomach sample contents to the fixed $12 per sample we estimated for determining stable isotope values. For stable isotopes, we then evaluate the relationship between isotopic value precision (95% CI), costs, and sample size using the data from the previous analysis. Minor differences or error in cost will have little influence on the results, as all comparisons are relative.

Results

Variability in stomach contents

Invertebrates were numerically dominant in the stomachs of all three salmonid species (Table 1). Amphipods, primarily *Hyalella azteca*, were most abundant and found in nearly all individuals, followed by Diptera, Ephemeroptera, Mollusca, terrestrial insects, other miscellaneous aquatic invertebrate taxa, and Coleoptera. Fish remains were found in 39% of the brown trout and 22% of the rainbow trout, and deer mice were found in 7% of the brown trout sampled. Salmonid eggs were found in 1% of the brown trout and 3% of rainbow trout. No fish or fish eggs were found in mountain whitefish, and no mice were found in rainbow trout or mountain whitefish. Three percent of the brown trout, 3% of rainbow trout, and none of the mountain whitefish had empty stomachs. Brown trout and rainbow trout stomach contents were more taxonomically diverse than mountain whitefish. Based on abundance, 91% of the items found in mountain whitefish stomachs were Diptera (49%) or Amphipoda (42%). Two large brown trout (471 and 510 mm) had only fish in their stomachs. The smallest fish that had consumed a fish was a 250 mm rainbow trout (the minimum size threshold for fish used in our analysis).

Results from the MDS ordination indicated that stomach contents did not group strongly by species or year (Fig. 1), suggesting little diet specialization among species or variation in diets among years, at least in September. Note that diet specialization could not be evaluated for other months of the year, as stomach contents were only examined for fish caught in September. This pattern was also confirmed by ANOSIM. Species groups nested within years were not significantly different from each other ($R = 0.02$, $p = 0.357$; pairwise R values = 0.08–0.112, pairwise p values = 0.127–0.206), indicating that stomach contents were virtually indistinguishable between salmonids. Similarly, stomach contents were similar among years ($R = 0.02$, $p = 0.357$; Fig. 1). The greater dispersion of data (Fig. 1) for brown trout relative to the other two species is likely an artifact of the greater number of individuals evaluated (see sample sizes). SIMPER did identify some minor differences; fish and mice, for example, were more abundant in brown trout, and Diptera and Ephemeroptera were more abundant in mountain whitefish.

Diet overlap versus sample size

Schoener diet overlap values were 0.61 between brown trout and mountain whitefish, 0.78 between brown trout and rainbow trout, and 0.81 between mountain whitefish and rainbow trout, suggesting biologically significant diet overlap among all three species (Zaret and Rand 1971). Based on our random bootstrap, mean diet overlap values among all species increased with increasing sample size before reaching a plateau around $n = 10$ for all comparisons. Precision around mean overlap values was consistently less (wider 95% CI) for sample sizes less than $n = 25$ for all comparisons (Fig. 2).

Variability in stable isotopes

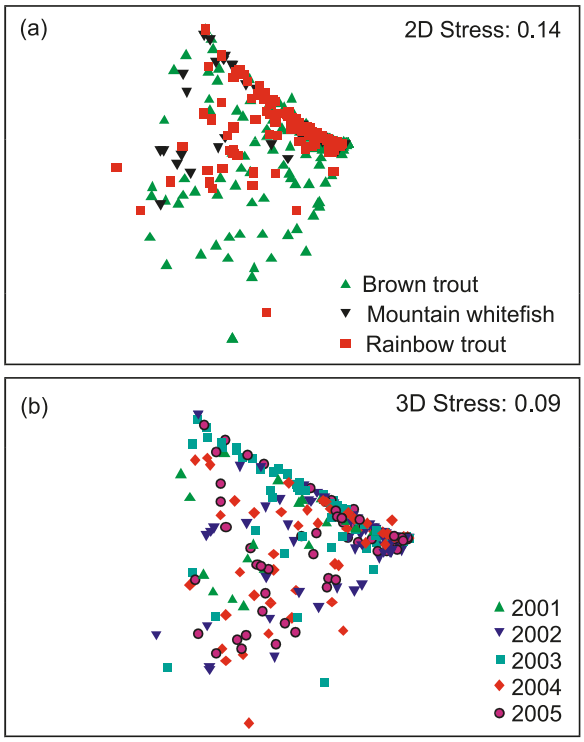
For both $\delta^{13}C$ and $\delta^{15}N$, we observed no significant variation in $\delta^{13}C$ and $\delta^{15}N$ measured from replicate muscles samples taken from an individual fish (see ±95% CI) and little

Table 1. The percent composition as occurrence (*%O*) and number of individuals (*%N*) in the stomachs of three salmonid species collected from the Green River downstream from Flaming Gorge Dam, Utah, annually in September from 2001 to 2005.

	Brown trout (n = 139)		Mountain whitefish (n = 33)		Rainbow trout (n = 111)	
Taxon	*%O*	*%N*	*%O*	*%N*	*%O*	*%N*
Ephemeroptera	41.6	6.2	60.8	7.5	50.4	8.0
Coleoptera	5.2	0.1	25.5	0.1	7.9	0.1
Diptera	79.2	10.3	100.0	49.1	96.1	20.8
Amphipoda	89.6	69.0	98.0	42.3	96.1	66.4
Mollusca	33.1	12.0	41.2	0.5	29.9	1.5
Other aquatic insects	24.7	0.7	41.2	0.5	42.5	1.0
Terrestrial insects	27.9	0.9	17.7	0.0	55.1	2.0
Fish eggs	1.4	0.1	0.0	0.0	2.7	0.1
Fish remains	39.0	0.6	9.8	0.0	22.1	0.1
Deer mice	7.1	0.1	0.0	0.0	0.0	0.0
Empty	3.2	—	0.0	—	3.1	—

Note: Data by salmonid species were pooled across years.

Fig. 1. Nonmetric multidimensional scaling ordination of diet categories among brown trout, mountain whitefish, and rainbow trout (*a*) and among 5 years of collection (*b*). Stomach contents were collected annually from the Green River downstream from Flaming Gorge Dam, Utah, in September between 2001 and 2005. Each point represents the stomach contents of an individual fish. Points close to each other represent similar stomach contents, and points more distant indicate differing contents.



variation between adipose tissue and muscle tissue. On average, the difference (absolute value) between pairwise comparisons of means among individual muscle tissue was greatest for rainbow trout (2.5‰ for δ^{15}N and 1.61‰ for δ^{13}C), followed by brown trout (1.5‰ for δ^{15}N and 1.1‰ for δ^{13}C) and whitefish (0.4‰ for δ^{15}N and 1.1‰ for δ^{13}C). There were no significant differences among any pairwise comparison between adipose and muscle tissue for either δ^{13}C or δ^{15}N for both rainbow trout and brown trout (df = 3–7, $P > 0.05$). For mountain whitefish tissue, we observed no statistical difference in mean δ^{15}N between adipose versus muscle (df = 6, $P > 0.05$); whereas, muscle tissue δ^{13}C was ~2‰ lighter than adipose δ^{13}C on average, a difference that was statistically significant (df = 7, $P = 0.003$). However, it is important to note that the variance was extremely low among replicate samples within each category, such that very small differences could be statistically, albeit not biologically, different.

Variation among individuals and precision versus sample size

The top model for both δ^{15}N and δ^{13}C included significant effects of fish species and year and fish length for δ^{15}N (Table 2). Across all years, mean δ^{15}N was significantly greater in brown trout (17.58‰) than in rainbow trout (16.82‰), but not between brown trout and mountain whitefish (17.18‰) or between mountain whitefish and rainbow trout. δ^{13}C was significantly less in mountain whitefish (–28.89‰) than in brown trout (–26.77‰) and rainbow trout (–27.25‰), but not between brown trout and rainbow trout. The range in mean δ^{15}N among years was 1.9‰ for brown trout, 2.0‰ for mountain whitefish, and 2.6‰ for rainbow. The range in mean δ^{13}C among years was 0.7‰ for brown trout, 2.1‰ for mountain whitefish, and 1.1‰ for rainbow trout. δ^{15}N increased significantly with length for all species. Brown trout and mountain whitefish δ^{15}N increased the least, and rainbow trout δ^{15}N increased the most, with variation (increase) in length (Fig. 3), indicating that rainbow trout appeared to undergo the greatest ontogenetic change in diet even after reaching 250 mm. Mountain whitefish δ^{13}C did not vary with fish length, rainbow trout δ^{13}C became significantly more depleted with increasing length, and brown trout δ^{13}C became significantly more enriched in larger fish (Fig. 3). The overall relationships between fish length and δ^{15}N and δ^{13}C values suggest diet

Fig. 2. Mean (±95% confidence interval, CI) Schoener diet overlap values (Schoener 1970) between brown trout and mountain whitefish (●), brown trout and rainbow trout (○), and mountain whitefish and rainbow trout (▼) for different samples sizes. Diet overlap is generally considered to be biologically significant when the value exceeds the horizontal line at 0.60 (Zaret and Rand 1971). Stomach contents were collected annually from the Green River downstream from Flaming Gorge Dam, Utah, in September between 2001 and 2005. Mean and 95% CI were calculated by randomly resampling 100 times for each sample size bin.

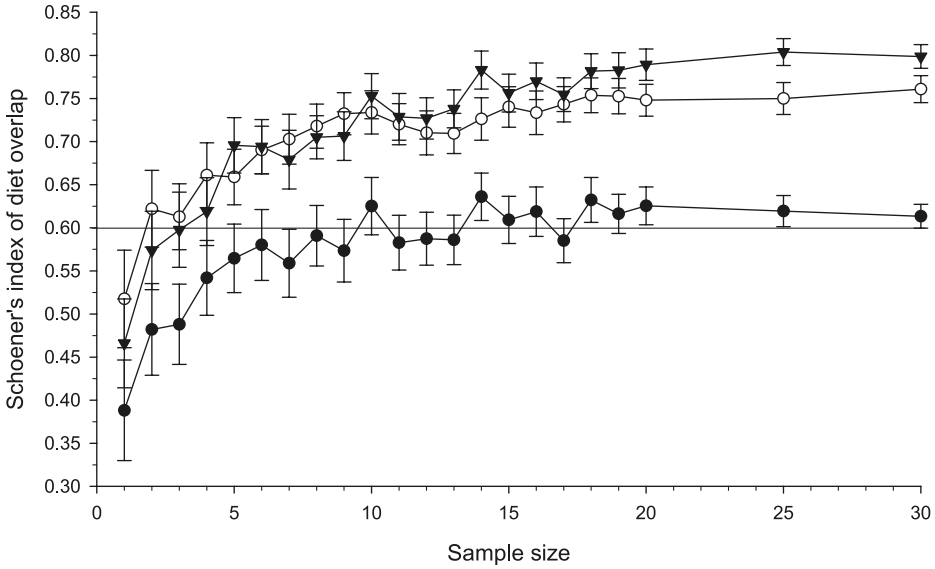


Table 2. Results for the general linear models of $\delta^{15}N$ and $\delta^{13}C$ as response variables.

Effect	Numerator df	Denominator df	*F*	*p*
$\delta^{15}N$				
Species	2	111	12.65	<0.0001
Year	4	131	39.26	<0.0001
Species × year	8	137	1.18	0.3170
Fish length	1	85.6	44.91	<0.0001
Fish length × species	2	93.1	5.56	0.0052
$\delta^{13}C$				
Species	2	89.9	14.99	<0.0001
Year	4	112.0	15.05	<0.0001
Species × year	8	131.0	1.52	0.1559
Fish length	1	72.7	0.18	0.6712
Fish length × species	2	91.1	15.30	<0.0001

Note: Fish species and year were included as categorical fixed-effects factors, and fish length was included as a centered, continuous fixed-effect factor.

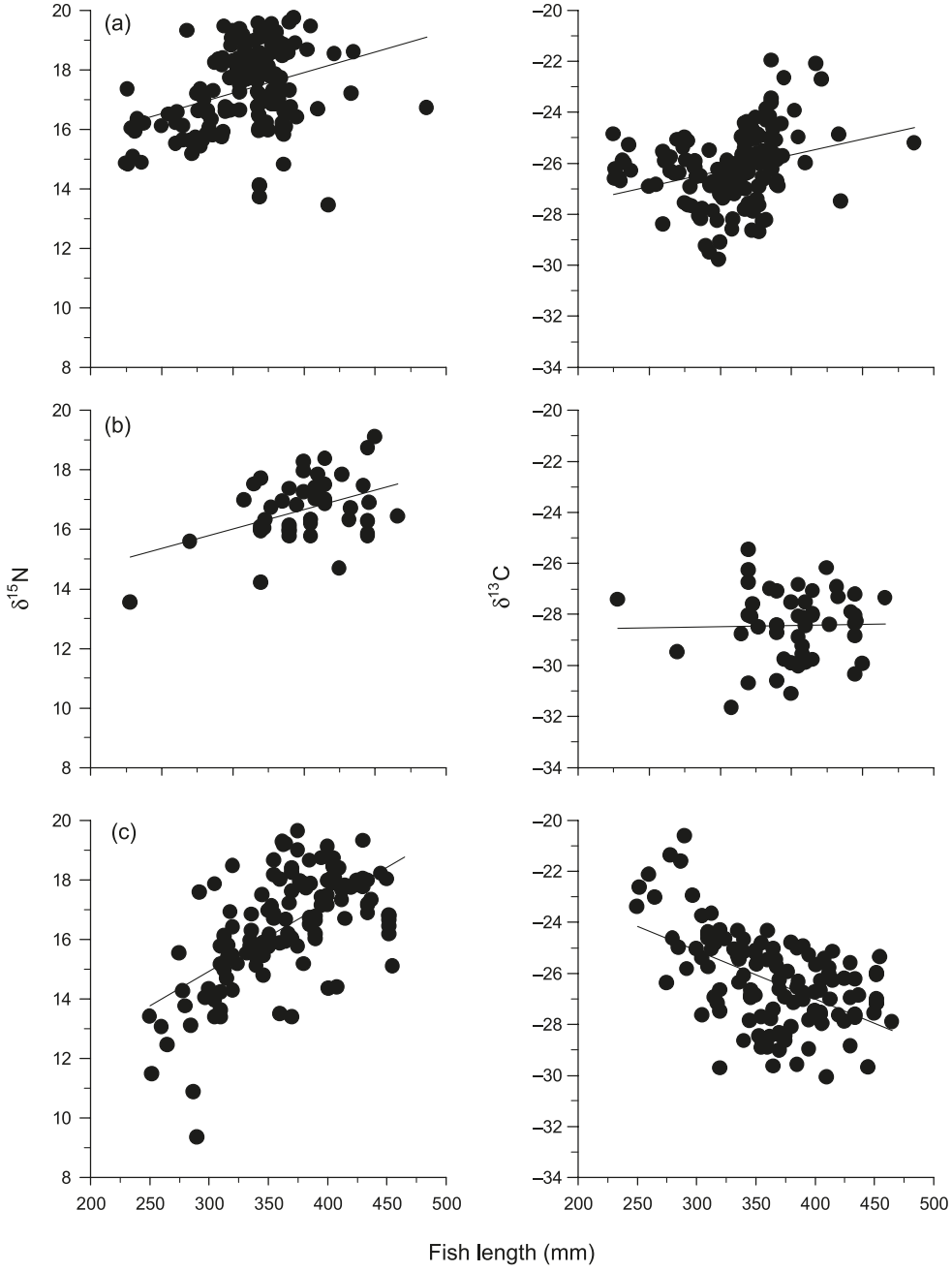
overlap was greatest between >350 mm brown and rainbow trout, and less overlap was apparent in smaller individuals.

Based on our bootstrapping simulations, the precision (95% CI) around the average $\delta^{13}C$ and $\delta^{15}N$ isotopic value increased substantially above sample sizes of n = 10 and reached a plateau around n = 15 for mountain whitefish, n = 20 for brown trout, and n = 25 for rainbow trout (Fig. 4). Precision for the average isotopic value was consistently less (wider 95% CI) for $\delta^{13}C$ as compared with $\delta^{15}N$ for all three species.

Food web and trophic overlap

Brown trout were the top predators, as indicated by their high $\delta^{15}N$ (Fig. 5). The mean $\delta^{15}N$ of mountain whitefish and rainbow trout were about 0.5–0.8‰ lighter than brown trout, respectively. The mean $\delta^{15}N$ of sculpin was 3.5‰ lighter than brown trout and 2.8‰ lighter than rainbow trout, suggesting brown trout prey heavily on sculpin and that rainbow trout also likely fed on sculpin. Mountain whitefish had similar mean $\delta^{15}N$ values as brown trout and rainbow trout, but their $\delta^{13}C$ was about 3‰ lighter, suggesting mountain whitefish rely on prey utilizing different basal resources. $\delta^{13}C$ values implied they were primarily consuming invertebrate detritivores; however, mean $\delta^{15}N$ for invertebrate detritivores was 7‰ less than mean mountain whitefish $\delta^{15}N$, indicating they were consuming and assimilating higher trophic level prey than invertebrate detritivores, such as salmonid eggs, which had mean $\delta^{15}N$ values 1.7‰ lighter than mountain whitefish (Fig. 5). Among invertebrates, predators occupied a higher trophic position than detritivores and herbivores. Mean $\delta^{13}C$ values for inver-

Fig. 3. Relationship between $\delta^{15}N$ and $\delta^{13}C$ values and fish length for (*a*) brown trout, (*b*) mountain whitefish, and (*c*) rainbow trout collected annually from the Green River downstream from Flaming Gorge Dam, Utah, in September between 2001 and 2005.



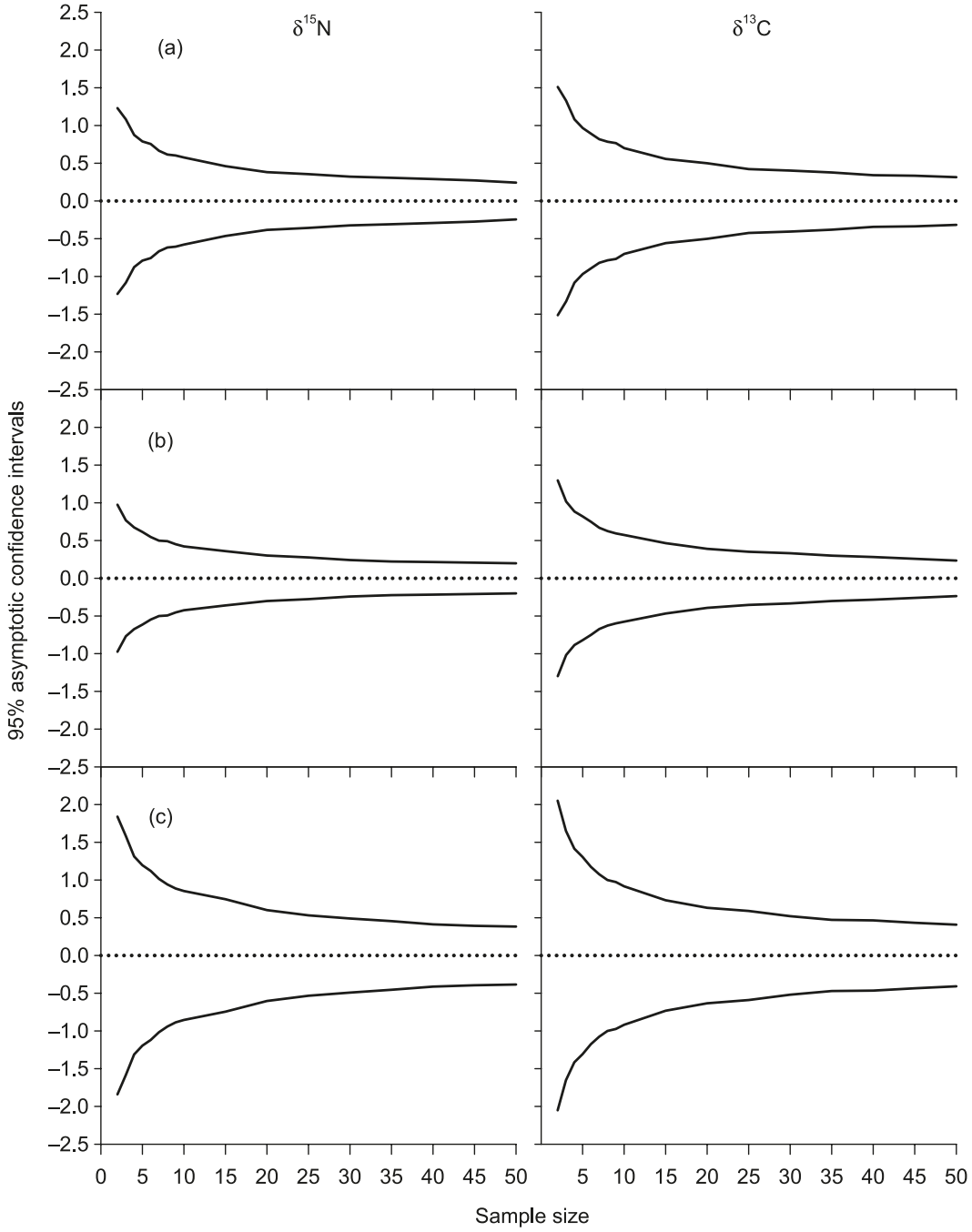
tebrate detritivores were 1.2‰ to 1.7‰ lighter than invertebrate predators and herbivores, indicating that invertebrate detritivores and herbivores were utilizing different resources.

Our analysis of the effect of sample size on trophic separation showed that major trophic level groups, i.e., predatory fish, prey fish, and invertebrates, and among salmonid species demonstrated distinct combinations of mean $\delta^{13}C$ and $\delta^{15}N$, but 95% CIs were broadly overlapping for sample sizes $n \leq 10$ (Fig. 5), suggesting little separation in dietary resources among trophic level groups when few samples were collected. Separation among trophic levels and among salmonid species increased with increasing sample size. For sample sizes $n \geq 10$, overlap in 95% CI among taxonomic or trophic groups was less evident, suggesting dietary resources became more distinct among trophic levels groups and especially between brown trout and rainbow trout when sample sizes reached $n = 25$.

Costs

Stomach content processing costs averaged \$25.49 ± \$2.91 (95% CI) and ranged from \$1.50 for an empty stomach to \$291.50 for a sample with 2330 items. The median cost was \$19.00, 36% more than the per-sample cost for determining $\delta^{15}N$ and $\delta^{13}C$ values. The average time to remove the debris and identify the organisms in a stomach sample was 1.3 ± 0.2 h (95% CI) and ranged from 0.1 to 13.5 h;

Fig. 4. The precision (95% confidence interval, CI) around mean $\delta^{15}N$ and $\delta^{13}C$ values from muscle tissue from (*a*) brown trout, (*b*) mountain whitefish, and (*c*) rainbow trout collected annually from the Green River downstream from Flaming Gorge Dam, Utah, in September between 2001 and 2005. 95% CIs were calculated using 1000 bootstraps of the data for each sample size category (increments of 1 for n = 2–10, increments of 5 for n = 10–50).



the median time was 1.0 h. The average time to enter the data into our computer program was 0.1 h per sample. Stomach sample processing costs increased sharply with the number of organisms within a stomach (Fig. 6).

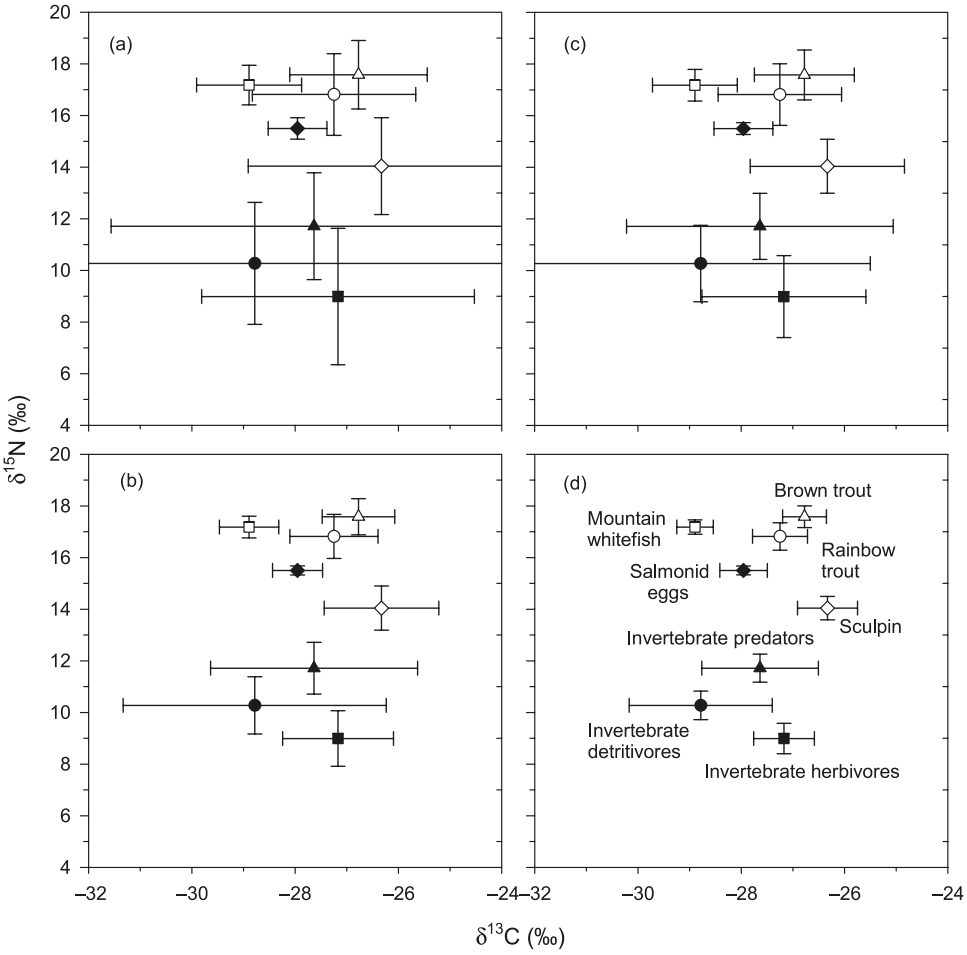
Based on our analysis of stable isotope costs and the natural variability around mean values, overall cost per analysis was minimized and precision was maximized at a sample size of n = 9 for $\delta^{15}N$ and n = 10 for $\delta^{13}C$ (Fig. 7). Precision (95% CI or standard error) increased dramatically up to a sample sizes of n = 10 (see also Fig. 4), while analysis costs increased linearly, indicating a sample size of around 10 generally provided the greatest precision for the lowest cost.

Discussion

Stomach content and stable isotope comparability

The general feeding habits of brown trout and rainbow trout were apparent using either stomach content or stable isotope data, but the overall conclusions varied when the results of each method were considered independently. For ex-

Fig. 5. Trophic structure of the Green River downstream from Flaming Gorge Dam, Utah, based on mean (±95% confidence interval, CI) $\delta^{13}C$ and $\delta^{15}N$ values derived from samples sizes of (*a*) 3, (*b*) 5, (*c*) 10, and (*d*) 25 for each trophic category. For the lower trophic levels (sculpin, salmonid eggs, invertebrate predators, detritivores, and herbivores), the mean was held constant at a sample size of 25 (as the purpose here is to show the effect of increasing precision), and the 95% CIs were calculated based on the mean standard deviation of 100 random drawn samples for each sample size. Mean and 95% CI for brown trout, rainbow trout, and whitefish are based on the bootstrap analysis. The data point symbols are the same for each plot and labeled in the n = 25 plot (*d*).



ample, MDS and Schoener (1970) diet overlap analyses of stomach content data both suggested there was less diet specialization and broad diet overlap among the salmonids species evaluated here and no significant differences in diet across years, at least during September. Conversely, analysis of stable isotope signatures suggested there were significant differences in species and across years, with the three species actually holding different trophic positions. These findings corroborate those of previous studies (e.g., Beaudoin et al. 1999; McIntyre et al. 2006; McHugh et al. 2008), which have generally found that among ecologically similar species, stable isotope data appears to show less dietary overlap among species than stomach content data. Clearly, when considered independently, both the implications for future study design and the ecological conclusions that one would draw from these two types of studies are complementary, yet may be substantially different.

In addition to these general differences in conclusions about feeding habitat that arise from the two different techniques, both methods provided important dietary insights that the other did not. Stomach content data provided information about trophic links that would have been undetectable if only stable isotope data had been evaluated, such as the fairly common consumption of deer mice by brown trout. Deer mice had mean $\delta^{15}N$ and $\delta^{13}C$ values of 3.69‰ and –23.96‰, respectively, which places them about four trophic levels beneath brown trout. As such, if we had not observed mice in stomachs, we would not have identified this terrestrial and aquatic food web connection. Conversely, trophic distinction, the amount of piscivory, and the relationship between increasing piscivory and fish size were much more readily observed using stable isotope data than stomach content data. $\delta^{15}N$ values for brown trout and rainbow trout were about 3‰ heavier than that observed in invertebrate-consuming sculpin and juvenile (<250 mm) trout (mean $\delta^{15}N$ = 14.9, n = 21), suggesting these fish consume more fish than that suggested by stomach content analysis alone. The opposing direction of the relationship of fish size and $\delta^{13}C$ between brown trout and rainbow trout was intriguing and suggests that as they grow even larger than the minimum size at which piscivory begins (~250 mm), they exhibit different foraging behavior and habitat use

Fig. 6. Laboratory processing costs associated with processing fish stomach content and stable isotope samples. The cost for processing stable isotope samples was a fixed \$12 per sample for sample preparation and $\delta^{13}C$ and $\delta^{15}N$ analysis costs. Costs associated with processing stomach samples varied strongly with the number of organisms present. Two out-of-range stomach sample processing costs were not shown, \$231.50 and \$291.50.

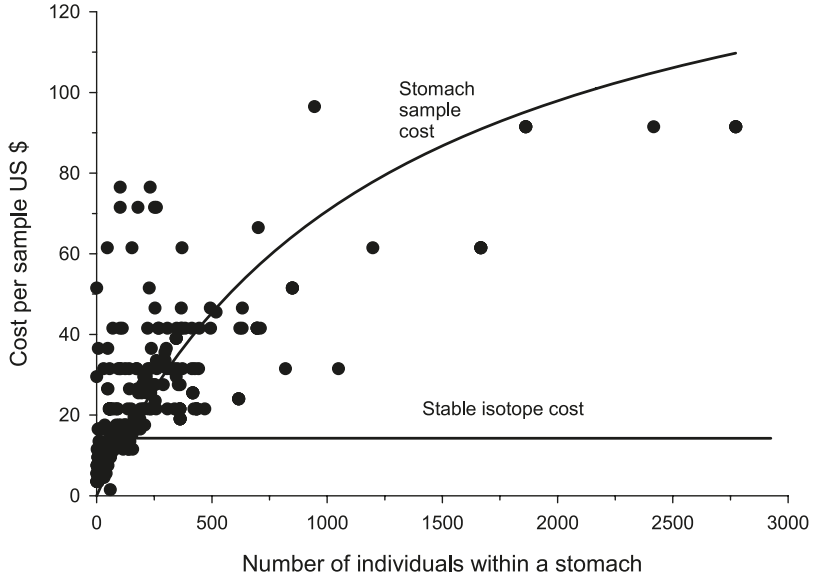
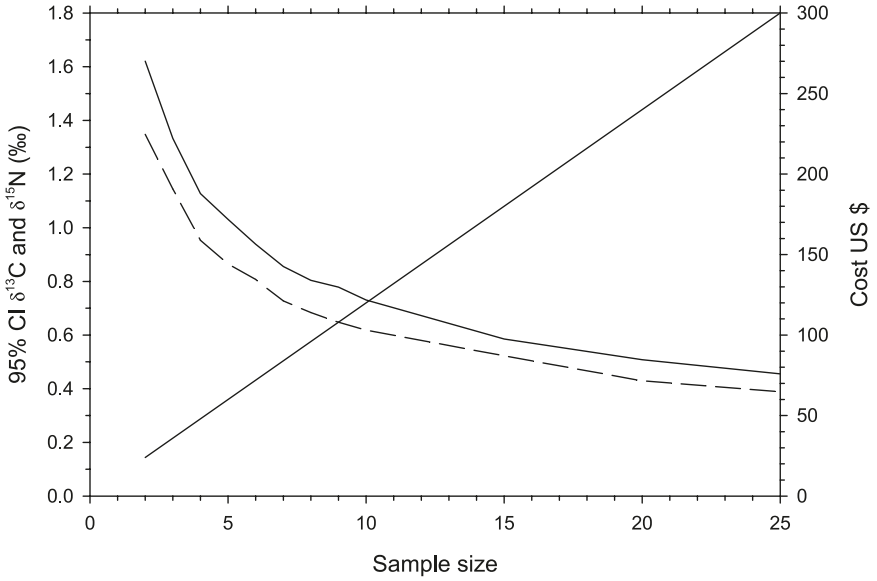


Fig. 7. The precision (95% confidence interval, CI) around pooled $\delta^{13}C$ (solid line) and $\delta^{15}N$ (dashed line) values from muscle tissue from brown trout, mountain whitefish, and rainbow trout and the costs associated with processing $\delta^{13}C$ and $\delta^{15}N$ samples (diagonal line, right *y* axis). Sample cost was \$12 per sample for sample preparation and to obtain both $\delta^{13}C$ and $\delta^{15}N$ values.



(Newsome et al. 2009). We found these results somewhat surprising given the relative simplicity of prey resources and habitat they occupy.

Comparative results between both techniques for mountain whitefish were less consistent than for the other two salmonids species; stomach contents indicated they ate invertebrate detritivores almost exclusively, whereas stable isotope data strongly suggested they were consuming higher trophic level organisms as well. Likely possibilities for alternative diet items at higher trophic levels include salmonid eggs or small fish, yet we found no fish or salmonid eggs in their stomachs. Stenroth et al. (2006) found similar conflicting results for crayfish (Decapoda: *Pacifastacus leniusculus*); $\delta^{15}N$ and $\delta^{13}C$ values suggested they were carnivores and stomach content data suggested they were detritivores. Mountain whitefish are well known to be bottom feeders that consume aquatic invertebrates, small fish, and fish eggs (Scott and Crossman 1973). The lack of salmonid eggs in our mountain whitefish stomachs may be due to either the rapid digestion of eggs (Bailey and Houde 1989) or sample size (i.e., we evaluated too few stomachs to detect eggs), or both. This scenario provides one example of the utility of using both techniques in parallel for food web studies; future analyses can now be targeted towards determining the un-

known source of heavier $\delta^{15}N$ values in mountain whitefish diets. In sum, combining the two approaches increases the ability to interpret the overall results and led us to conclude the three salmonid species consume similar prey, but in different proportions, leading to greater niche segregation than suggested by stomach content data alone.

Variation within and among individuals and inherent limitations

We observed no significant or influential differences in isotopic composition for either $\delta^{15}N$ or $\delta^{13}C$ within individual fish or between samples collected from muscle tissue versus adipose fins. These results corroborate recent work by Sanderson et al. (2009) and Hanisch et al. (2010) and indicate it is not necessary to analyze more than one isotopic sample per individual, and that for salmonid species (and likely most fishes with an adipose fin) sampling either muscle or fins will result in the same answer. These results have obvious and encouraging implications for isotopic analyses of imperiled or protected species, as removing the muscle plugs from adult fish, or adipose fins from any age fish, is neither highly invasive nor lethal.

For studies of naturally occurring stable isotopes, variance among individuals within a population is likely the highest source of variation encountered; however, it is also a source of variation that can be controlled by adjusting study design, sample sizes, and analyses (Focken and Becker 1998; Branstrator et al. 2000). First, variation in isotopic signatures can be reduced by stratifying sampling by season or year (Vizzini and Mazzola 2003; Matthews and Mazumder 2005; this study) or habitats (Genner et al. 2003) and then by grouping age or size classes where possible post hoc (Beaudoin et al. 1999; Fry et al. 1999; this study). In contrast, although variation in stomach content samples can be minimized to some degree by sampling during times when fish are known to be feeding (e.g., crepuscular times, nonmating times), not all fish will be feeding, and many are likely to have empty stomachs or partially full stomachs (Arrington et al. 2002). The evaluation of the relative importance of individual diet items may also be skewed by both the short temporal window over which stomach content samples are generally collected and whether the sampling coincides with the occurrence of a particular prey item on the day or time the samples were collected (e.g., highly seasonal emergence event for an aquatic insect population; Ephemeroptera: Ephemeridae, or migrating terrestrial invertebrates; cicadas; Insecta: Cicadidae).

Fish collection methods may have adverse effects on stomach contents while having no effect on fish tissue used for isotope analyses. For example, gillnet-caught fish may regurgitate their stomach contents prior to collection of their stomach contents, and certain sampling techniques may be biased by collection of potentially more active, hungrier individuals (e.g., angling). It can also be difficult to identify stomach contents, especially larval or juvenile fishes and eggs, which are digested extremely rapidly (Bailey and Houde 1989; Schooley et al. 2008). Much of the evidence for piscivory in stomach content analyses is based on partially digested, unidentifiable bones that require educated guesses as to the species, size, and number of fish consumed or genetic analyses (Sheppard and Harwood 2005). The information obtained via stomach content analysis can be rewarding, but may also be difficult and time consuming to obtain.

Although generally more precise than diet data, when sample sizes are adequate, stable isotope data are also subject to their own suite of limitations and sources of variation that may be underestimated in our study. First, our study was performed in a large mountain river, typical of the Intermountain West and was characterized by a relatively simple food web (e.g., four to five trophic levels and only four species of fish). Isotopic overlap will generally increase as the number of species and trophic levels increases in more complex food webs, thus necessitating a greater sample size per species evaluated and potentially limiting our ability to infer trophic status and niche differentiation; e.g., see Szepanski et al.'s (1999) analysis of wolf diet data reanalyzed by Phillips and Gregg (2003). Although not a focus of our study, isotopic mixing models that report results in terms of the distribution of possible results can reduce the effects of these complications (Phillips and Gregg 2003). However, the typically unknown failure to include all end members, small sample sizes, and necessary assumptions regarding fractionation still limit our ability to infer trophic status and habitat use when food webs are complex and prey sources occupy numerous trophic levels. Nevertheless, additional research on species-specific isotopic turnover rates and fractionation, currently an area of considerable focus in stoichiometry and biogeochemistry, will likely improve the predictability of isotopic signature transfer between organisms.

Study design lessons and recommendations

While we provide specific recommendations on study design and sample size for ecological isotopic composition studies below, the rule for determining sample sizes should be similar to the general rule for all statistical procedures. Sample sizes must be scaled to the level of detection required to address study hypotheses; the smaller the difference that must be detected, the larger the sample size must be (Krebs 1989). These optimizations are more difficult in stomach content studies where measures of variation are difficult to acquire. Ferry and Cailliet (1996) reported that none of the more than 200 dietary studies they reviewed provided any estimates of precision in describing diets. We also had difficulty in determining a useful measure of variance for our stomach content data, and in our analysis, diet overlap increased with increasing sample sizes, a trend opposite of that inferred from stable isotopes.

Our study of stomach contents and stable isotope data from the Green River is one of just a few field studies that have applied these two approaches to the same fish (Beaudoin et al. 1999; McIntyre et al. 2006; McHugh et al. 2008). Collectively, these studies have found that stable isotope data perform better at identifying trophic position and ontogenetic changes in diet, while stomach content analyses perform better at identifying specific prey species consumed. We believe the recommendations we provide below will improve future studies and increase standardization of study design with respect to sample sizes. While our analysis of 283 fish for both isotopes and diet was quite large, we note that this data was collected from a relatively simple, albeit common food web including only four to five major trophic

levels and only four species of fish; our recommendations should be taken within the context of that data set.

Based on stomach contents, even with a very large data set, we could not readily or statistically distinguish differences in stomach contents among species or years, but we did obtain a reasonable qualitative sense of which prey were being consumed by fish; this information helped elucidate some of the patterns we observed in the stable isotope analysis. Observing wide variance in diets among individuals, while not statistically pleasing, does have important implications for understanding the overall ecology of the system and population dynamics of both predators and prey. Another consideration for collecting stomach content samples will be when comparisons to historical (before-isotope) diet information are needed. By combining stable isotope and stomach content analyses, we concluded the three salmonid species consume similar prey, but in different proportions. These results support the generally held view that stable isotopes provide a time-integrated view of diets, whereas stomach contents provide a snapshot of recently digested prey and prey availability at that time. Depending on the question and the desired temporal inferences, a combination of the two approaches may be most appropriate.

Based on our bootstrapping exercise, precision in $\delta^{15}N$ or $\delta^{13}C$ increased dramatically up to a sample size of $n = 10$, with considerably less increase in precision as sample size increased further. In general, we could identify differences among all trophic levels and three ecologically similar fish species at a sample size of $n \geq 10$ and could identify significant differences at $n \geq 25$. Based on these results, stable isotope samples sizes between 10 and 25 are probably adequate for addressing most ecological questions; however, a slightly greater n may be required for addressing questions where carbon is the sole or primary focus or for species with more expansive diets, e.g., in our study, characterization of rainbow trout diets would require more samples than would mountain whitefish. Stenroth et al. (2006) recommended a sample size of 14 for detecting a 1‰ difference in isotopic values between crayfish populations. For stomach content analyses, mean diet overlap values among all three species plateaued around $n = 10$, and variation around mean values (95% CI) was consistently less (wider 95% CI) for sample sizes less than $n = 25$ for all comparisons. These results suggest that samples sizes of $n \geq 25$ are probably needed for addressing diet overlap among ecologically similar species via stomach content analysis.

In terms of cost, isotope-associated costs are known precisely prior to project implementation, whereas stomach content analysis costs vary with stomach fullness and level of taxonomic detail. In our study, isotopes averaged about one-third of the cost of stomach content analyses. The overall cost per isotope analysis was minimized, and precision was maximized, at a sample size of $n \sim 10$ for both $\delta^{15}N$ and $\delta^{13}C$. Beyond this sample size ($n \geq 10$), precision increased at a much slower rate while costs increased linearly, again indicating a sample size of about $n \geq 10$ generally provides the most precise information for the given cost. Note however, this determination will be dependent on the similarity in diet between species; for example, in our study, the ability to differentiate trophic status between brown trout and mountain whitefish would require fewer samples ($n \sim 5$) than it would to differentiate between brown trout and rainbow trout ($n \sim 25$). Lower costs associated with stable isotopes may allow for increased stratification by fish size, habitat, and season that may help explain the observed variance among individuals. Nevertheless, for stable isotopes, one also may need to collect data for prey that likely contribute to the diet, and it may be necessary to obtain these samples more than once a year, as the temporal variation in stable isotope signatures tend to increase with decreasing body size (Cabana and Rasmussen 1996). High costs associated with processing stomachs with large numbers of prey items can be reduced through subsampling, but subsampling may also reduce the ability to detect rare prey items (Vinson and Hawkins 1996). In conclusion, both approaches are complementary and appear to work best when they are combined together. Therefore, the true cost of obtaining diet information could be higher when using either alone.

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